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Leafy Gall Formation Is Controlled by *fasR*, an AraC-Type Regulatory Gene in *Rhodococcus fascians*

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Rhodococcus fascians **can interact with many plant species and induce the formation of either leafy galls or fasciations. To provoke symptoms, *R. fascians* strain D188 requires pathogenicity genes that are located on a linear plasmid, pFiD188. The *fas* genes are essential for virulence and constitute an operon that encodes, among other functions, a cytokinin synthase gene. Expression of the *fas* genes is induced by extracts of infected plant tissue only. We have isolated an AraC-type regulatory gene, *fasR*, located on pFiD188, which is indispensable for pathogenesis and for *fas* gene expression. The combined results of our experiments show that in vitro expression of the *fas* genes in a defined medium is strictly regulated and that several environmental factors (pH, carbon and nitrogen sources, phosphate and oxygen content, and cell density) and regulatory proteins are involved. We further show that expression of the *fas* genes is controlled at both the transcriptional and the translational levels. The complex expression pattern probably reflects the necessity of integrating a multitude of signals and underlines the importance of the *fas* operon in the pathogenicity of *R. fascians*.**

The gram-positive bacterium *Rhodococcus fascians* (58) infects diverse plant species. Infection of dicotyledonous plants can result in the local proliferation of meristematic tissue, leading to galls that are covered with leaflets, known as leafy galls (17, 61). On monocotyledonous plants, such as lilies, *R. fascians* provokes severe malformations of the bulbs and the formation of long side shoots (37, 60), resulting in abnormal plants that are unfit for commercial use (2, 18). Infection of tobacco seedlings with *R. fascians* strongly inhibits growth, accompanied by arrested root development, thickening and stunting of the hypocotyl, and inhibition of leaf formation (10).

In 1966, the production of cytokinins was inferred as a major virulence determinant of *R. fascians* (31, 57). In our laboratory, in *R. fascians* strain D188, genes involved in pathogenicity were shown to be located on a large, conjugative, linear, fasciation-inducing plasmid (pFiD188) (10). Random mutagenesis of pFiD188 led to the identification of three virulence loci, of which the best characterized is the essential *fas* locus. This locus consists of an operon of six genes, of which the most important are a cytochrome P450 homologue gene (ORF1) and an isopentenyl transferase (*ipt*) gene (ORF4) homologous to *ipt* genes of other phytopathogens (10, 11). The *ipt* genes are typically involved in the biosynthesis of isopentenyl AMP (i^6AMP), a general precursor of several cytokinins (29). However, the chemical structure of the compound resulting from the action of the *fas* gene products remains to be determined. Two other pFiD188-located virulence loci, *hyp* and *att*, are necessary for balanced virulence because mutations in these regions result in hypervirulence and attenuated virulence, respectively (10).

Expression of the *fas* genes is induced by extracts of infected plant tissues and not of uninfected plants (10). In many other pathogens, induction of a whole battery of virulence genes follows sensing of signals from the environment (5, 9, 38, 51, 54, 65). This environmentally modulated expression is often mediated by a single pleiotropic regulatory protein (21, 28) or by a two-component regulatory system (27, 55).

Here, we report on the isolation and characterization of a new virulence gene located on pFiD188 that codes for a regulatory protein belonging to the AraC family (21, 49). We present data on the significance of this gene for *R. fascians* pathogenesis on tobacco and reveal its involvement in the complex regulation of *fas* gene expression.

Nucleotide sequence accession numbers. The sequence determined in this study has been deposited in the EMBL database (accession no. Y09820).

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used are listed in Tables 1 and 2. *Escherichia coli* strains were grown at 37°C in Luria broth (50), whereas *R. fascians* strains were grown at 28°C in yeast extract broth (YEB) (39). For determining *fas* gene expression levels, *R. fascians* strains were grown in MinA medium [6.4 mM KH_2PO_4, 33.6 mM K_2HPO_4, 0.1% $(NH_4)_2SO_4$, 0.05% sodium citrate, 0.025% $MgSO_4$, 0.001% thiamine, and 20 mM carbon source of interest]. When appropriate, media were supplemented with carbenicillin (200 μg/ml), chloramphenicol (25 μg/ml), or phleomycin (1 μg/ml).

DNA sequencing and analysis. The DNA sequence of both strands was determined by using automated dideoxy-sequencing systems (A.L.F. DNA Sequencer [Pharmacia, Uppsala, Sweden] and ABI377 DNA Sequencer [Applied Biosystems, Foster City, Calif.]). Computer-assisted interpretation of the sequence was performed by the Genetics Computer Group (Madison, Wis.) sequence analysis software package (version 9.1). Homology searches with the Swiss-Prot (release 35), Unique-PIR (release 53), and EMBL (release 53) databases were done using the FASTA algorithm (44). Alignments were done using PILEUP.

Deletion mutagenesis. The *fasR* deletion mutant was isolated via double homologous recombination. For this purpose, plasmid pUCDV3 was constructed; it carries the chloramphenicol resistance (*cmr*) gene (15) and the DNA region containing *fasR*, in which a deletion was generated (Fig. 1). Because pUCDV3 cannot replicate in *R. fascians*, electroporation into strain D188 and plating on chloramphenicol-containing medium resulted in the isolation of single recombinants. Growth of these clones without selective pressure allowed a second recombination event, and after screening was performed for Cm^s transformants, a deletion mutant was isolated. First and second recombinations were verified by Southern hybridization analysis (50).

Virulence tests. Sterile *Nicotiana tabacum* (L.) W38 seeds were germinated on half-strength MS medium (42) supplemented with 0.001% thiamine, 1% sucrose, and 0.8% agar. For the virulence assays, after 2 days of germination, when the

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TABLE 1. Bacterial strains

Strain	Description	Reference
E. coli		
MC1061	*ara*Δ*139* (Δ*ara leu*)*7697* Δ*lacX74 galU galK hsrR hsrM*$^+$ *rpsL*$^+$ StrA	7
DH5α	F$^-$ ϕ80d*lacZ*ΔM15Δ (*lacZ4A argF*)*U169 recA1 endA1 hsdR17* (r_K^+ m_K^+) *supE44* λ$^-$ *thi gyrA relA1*	26
R. fascians		
D188	Wild type; virulent	13
D188-5	Plasmid-free strain; nonpathogenic	13
D188Δ*fasR*	Deletion mutant; nonpathogenic	This work

radicle emerged, 20 μl of a concentrated *R. fascians* culture was added to the seedlings, or the plants were decapitated and infected with a saturated *R. fascians* culture 6 to 7 weeks after germination. Phenotypes were scored after 2 to 4 weeks.

Inductions and GUS assays. For the in planta expression analysis, 3- to 4-week-old sterile *N. tabacum* W38 plants were immersed in a culture of the test strain resuspended in MinA medium, and submitted to a vacuum generated by a water pump for 2 min. After being washed with MinA medium, the plants were replanted in half-strength MS medium, and after 3 days they were used for extraction and β-glucuronidase (GUS) measurements. Extracts were prepared by extensively crushing the plants or leafy galls excised from the infected plants with a pestle in an Eppendorf tube. After centrifugation and filter sterilization, a 50- to 70-μl extract was obtained from 100 mg of tissue. For the in planta expression assay, 1 ml of MUG buffer (50 mM $NaPO_4$ [pH 7.0], 10 mM β-mercaptoethanol, 10 mM Na_2EDTA, 0.1% sodium dodecyl sulfate, and 0.1% Triton X-100) was added to 200 mg of crushed plant tissue. The substrate 4-methylumbelliferyl-β-D-glucuronide (0.1 mM) was added, the reaction mixtures were kept at 37°C, and the reactions were stopped after 1 h by adding a 50-μl sample to 200 μl of 0.2 M Na_2CO_3. GUS activity was determined by excitation at 365 nm and measurement of emissions at 460 nm and is calculated as the measured emission × 1,000/time (in minutes). Every assay was performed on the same amount (fresh weight) of plant material, leading to relative and comparable data.

For *fas* and *fasR* gene expression, cells were grown for 2 days in YEB, diluted 10-fold in YEB, and allowed to grow overnight. After growth on YEB, the cells were collected by centrifugation, washed, and diluted to the desired optical density at 600 nm (OD_{600}) in MinA medium. The pH of the MinA medium was adjusted to 6.5 and 5.7 by changing the KH_2PO_4/K_2HPO_4 ratio and to 3.0, 4.0, 5.0, and 5.7 by using citric acid and sodium citrate as a buffer system (10 mM). GUS activity was measured after the cells were incubated overnight with gall extracts (20 μl/ml), tobacco plant extracts (40 μl/ml), different carbon sources (20

TABLE 2. Plasmids

Plasmid	Marker gene(s)	Relevant characteristics	Reference
pRF37	Phleo^r Ap^r	Shuttle cloning vector replicating in *R. fascians* and *E. coli*	14
pJGV131	Ap^r	Shuttle cloning vector replicating in *R. fascians* and *E. coli*	11
pUCDV1	Cm^r Ap^r	*Bam*HI clone containing a 4.9-kb upstream region of mutant *fas6* and the *cmr* gene as a 2.5-kb *Xba*I fragment in pUC18	11
pUCDV3	Cm^r Ap^r	Clone derived from pUCDV1 by deleting a 912-bp *Acc*I-*Nco*I fragment in the *fasR* gene, resulting in a suicide plasmid for the generation of D188Δ*fasR*	This work
pRFDV2	Phleo^r Ap^r	Clone of a 1.7-kb *Xho*I fragment of pFiD188 derived from *Bam*HI fragment 1 and containing the *fasR* gene in pRF37, resulting in a complementation construct for D188Δ*fasR*	11; this work
pJBDV1	Ap^r	Clone of the 0.9-kb *Xho*I fragment of pFiD188 *Bam*HI fragment 1 containing the *fasR* promoter and the 1.9-kb *Sal*I-*Sac*I fragment of pRG960sd containing *uidA* in pJB66, resulting in a transcriptional fusion between *fasR* and *uidA*	4, 59; this work
pRFDV6	Phleo^r Ap^r	Clone of the 2.8-kb *Xba*I fragment of pJBDV1 in pRF37, replicating transcriptional *fasR-uidA* fusion	This work
pJDGV2	Ap^r	Clone of the 1.5-kb *Stu*I-*Sno*I fragment of pFiD188 *Bam*HI fragment 1 in pGUS1, resulting in a translational fusion between the 111 amino-terminal amino acids of ORF1 of the *fas* operon and *uidA*	11
pJDGV3	Cm^r Ap^r	Clone of the 4.0-kb *Hin*dIII-*Xba*I fragment of pJDGV2 in pJGV131 with the 2.5-kb *Xba*I fragment containing the *cmr* gene of *R. fascians* NCPPB1675, replicating translational ORF1-*uidA* fusion	11
pJDGV4	Cm^r Ap^r	Clone of the 2.9-kb *Asc*I-*Xba*I fragment of pJDGV2 in pJGV131 with the 2.5-kb *Xba*I fragment containing the *cmr* gene of *R. fascians* NCPPB1675, replicating translational ORF1-*uidA* fusion	11
pJDGV5	Cm^r Ap^r	Clone of the 3.3-kb *Sal*I-*Xba*I fragment of pJDGV2 in pJGV131 with the 2.5-kb *Xba*I fragment containing the *cmr* gene of *R. fascians* NCPPB1675, replicating translational ORF1-*uidA* fusion	This work
pUCWT1	Cm^r Ap^r	Clone of the 4.0-kb *Hin*dIII-*Xba*I fragment of pJDGV3 in pUC18 with the 2.5-kb *Xba*I fragment containing the *cmr* gene of *R. fascians* NCPPB1675, integrating translational ORF1-*uidA* fusion	43; this work
pSPWT1	Ap^r	Clone of the 0.6-kb *Sal*I-*Sac*I fragment of pFiD188 *Bam*HI fragment 1 containing the *fas* promoter with the 2.1-kb *Sma*I-*Eco*RI fragment of pRG960sd containing the *uidA* gene in pSP72 (Promega, Madison, Wis.), resulting in a transcriptional fusion between ORF1 of the *fas* operon and *uidA*	59; this work
pJBWT1	Ap^r	Clone of the 2.5-kb *Pvu*II-*Bgl*II fragment of pSPWT1 in pJB66, integrating transcriptional ORF1-*uidA* fusion	4; this work
pRFWT11	Phleo^r Ap^r	Clone of the 2.5-kb *Xba*I-*Hin*dIII fragment of pJBWT1 in pRF37, replicating transcriptional ORF1-*uidA* fusion	This work
pJBWT2	Phleo^r Ap^r	Clone of the 3-kb *Bam*HI fragment of pMSA4 containing the Phleo^r gene in pJBWT1, integrating transcriptional ORF1-*uidA* fusion	56; this work

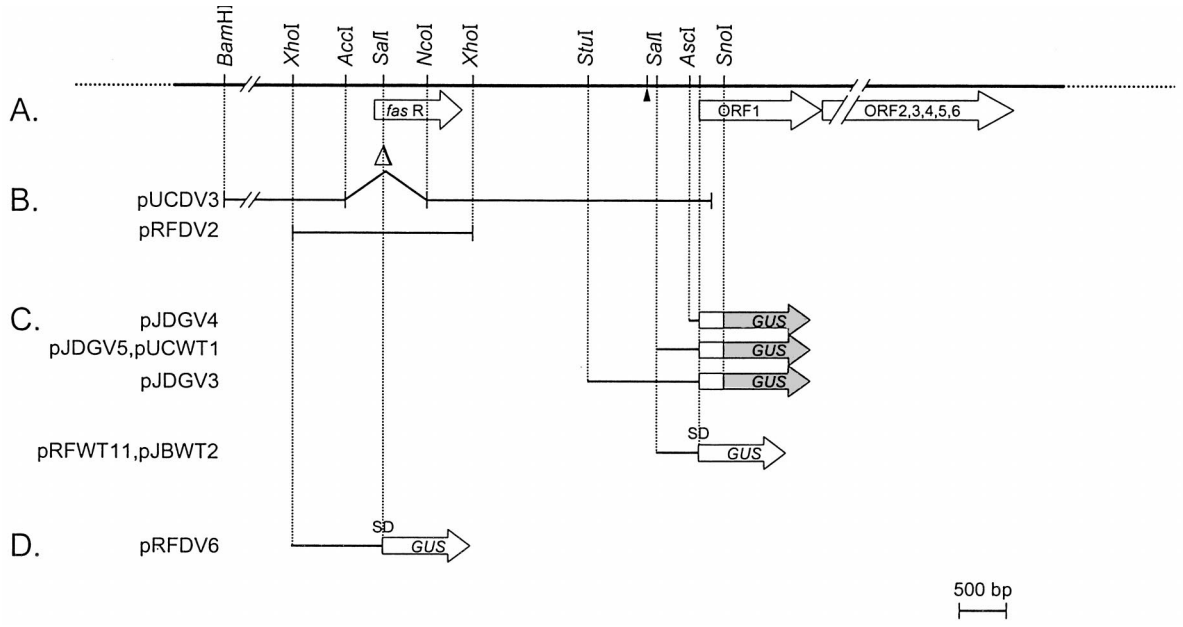

FIG. 1. Physical map of the relevant region of pFiD188. (A) Physical map of the region of pFiD188 spanning *fasR* and the *fas* operon. ORFs and relevant restriction sites are shown. The arrowhead indicates the previously determined 5′ border of the *fas* operon. (B) pUCDV3, suicide plasmid carrying a 912-bp *Acc*I/*Nco*I deletion (Δ) in the *fasR* region used to generate D188Δ*fasR*. pRFDV2 is a fragment used for the complementation analysis. (C) Fragments used for the *fas-gus* fusions. The shaded and open arrows represent translational and transcriptional GUS fusions, respectively. (D) Fragment used for the transcriptional *fasR-gus* fusion. SD, Shine-Delgarno sequence.

mM), and/or amino acids (5 mM). For the GUS assay, the cells were collected by centrifugation and resuspended in 1 ml of MUG buffer, and the GUS activity was measured as described above and calculated as the measured emission × (1,000/OD_{600}) × time (in minutes).

Other methods. Plasmid isolation and DNA cloning were performed according to the methods of Sambrook et al. (50), and *R. fascians* transformation was done as described before (14).

RESULTS

An AraC-type regulatory gene, *fasR*, is essential for virulence. Determination of the DNA sequence between the linked *fas* and *att* locus (10) revealed an open reading frame (ORF) of 834 bp (potentially encoding a protein of 277 amino acids) located 3,282 bp upstream from ORF1 of the *fas* operon and in the same transcriptional orientation (Fig. 1A). Three base pairs upstream from the ATG start codon, the sequence GAACGACAG, which represents a putative ribosome-binding site of *R. fascians*, is present (11). The ORF has a G+C content of 53% and a G+C content at the third position of 50%, both very low for *R. fascians* (G+C, 61 to 68%) (34). All codons are used in this ORF, but remarkably, UUA, which is usually a rare codon in *R. fascians* as well as in *Streptomyces* and corynebacteria (35, 66), is frequently used for Leu (7 out of 30).

Comparative sequence searches revealed that this ORF potentially encodes a protein that is homologous to different members of the AraC family of transcription regulators (22) (Fig. 2). Although the similarity of these proteins is highest in the carboxyl terminus, where the DNA-binding helix-turn-helix motifs are located, the overall similarity is also significant. Over a 100-amino-acid-residue stretch, encompassing the defined AraC family profile (PROSITE database entry PSO1124), the highest similarities are found with an AraC-type regulator involved in rapamycin biosynthesis in *Streptomyces hygroscopicus* (38% identity; 48% similarity) (41), with the transcription regulator (NitR) of the nitrilase gene of *Rhodococcus rhodochrous* (34% identity; 43% similarity) (33), and with MoaB, a positive regulator of the monoamine oxidase gene of *E. coli* (34% identity; 47% similarity) (68) (Fig. 2).

Because the ORF is located between two pathogenicity loci, *fas* and *att*, the possible role of this gene in the virulence of *R. fascians* was examined by deleting part of the ORF in pFiD188. For this purpose, plasmid pUCDV3 (Fig. 1B), which carried a 912-bp *Acc*I/*Nco*I deletion in the region, was introduced into the wild-type strain D188. Because this plasmid could not replicate in D188, selection for chloramphenicol resistance (Cm^r) followed by a subsequent screening for the loss of the vector-located marker gene (*cmr*) resulted in the isolation of homogenotes that carried a deletion in pFiD188, as judged by Southern hybridization analysis (data not shown). Inoculation of such a deletion mutant on tobacco seedlings and on decapitated tobacco plants showed that it was not pathogenic (Fig. 3). This phenotype was identical to the described *fas* phenotype (10), suggesting that the new ORF could control *fas* gene expression. Because of the infection phenotype and the relation of the ORF to a family of regulatory genes, the ORF was named *fasR* (for fasciation regulator), and the corresponding mutant strain was called D188Δ*fasR*. Introduction of a replicating plasmid, pRFDV2, covering *fasR* (Fig. 1B) in strain D188Δ*fasR* restored virulence (Fig. 3).

Expression of the *fas* locus is induced during interaction with the plant. Three replicating plasmids, pJDGV3, pJDGV4, and pJDGV5, that carry translational *uidA* (*gus*) fusions to the regions of the cytochrome P450 gene encoding the 111 amino-terminal amino acids (ORF1) and different lengths of the upstream region (Fig. 1C) were introduced into strain D188 via electroporation. Subsequently, the expression of ORF1 was determined in planta. For this purpose, 3-week-old tobacco plants were infected by vacuum infiltration with cultures of the wild-type strain and of the three recombinant *R. fascians* strains, and 3 days later, the GUS activity of extracts of the

infected plants was determined (see Materials and Methods). The GUS levels obtained with plasmids pJDGV3 and pJDGV5 were very high (416.2 ± 112.8 and 512.4 ± 9.2, respectively), whereas plasmid pJDGV4 showed no GUS activity (36.4 ± 25.7 compared to 61.7 ± 35.7 when no plasmid was present). These results show that the sequences located between the *Stu*I site of pJDGV3 and the *Sal*I site of pJDGV5 are not required for *fas* gene expression and narrow down the previously determined 5′-end border of the *fas* operon (11) by 105 bp (Fig. 1A). Because the expression levels of strains D188(pJDGV3) and D188(pJDGV5) were comparable, only plasmid pJDGV5 was used further in this study.

The next step was to monitor *fas* gene expression in vitro. Using D188(pJDGV5) as the test strain, the *fas* genes were shown not to be expressed in rich medium (Table 3) or in a defined medium (MinA) (data not shown). Also, the addition of plant extracts to MinA medium did not induce *fas* gene expression (Table 3). However, when leafy gall extracts were added to the medium, a 10-fold induction of expression was obtained. This result was in agreement with previous data showing that *ipt* gene expression was induced by extracts of leafy galls (10).

Environmental signals influence *fas* gene expression. To characterize the parameters that affect expression levels in vitro, the influence of pH, the presence of phosphate, carbon and nitrogen sources, cell density, and oxygen concentration were examined with and without the addition of leafy gall extract. First, the role of the pH of the MinA medium at the start of the induction was evaluated. The data given in Fig. 4A showed that the induction of ORF1 was much higher at lower pH, with a peak expression level at pH 5.0. For the setting of the desired pH, either phosphate or citrate buffers were used. Thus, it became clear that the expression of the *fas* genes was negatively influenced by the presence of phosphate. Indeed, the addition of different concentrations of phosphate to citrate-buffered MinA medium at pH 5.0 significantly decreased gall-dependent induction (Fig. 4B).

Next, at pH 5.0, the glucose in MinA medium was replaced by other carbon sources (20 mM). The results show that none of the tested compounds alone (data not shown) or in combination with plant extracts led to *fas* gene expression (Table 3). Some carbon sources had no effect on gall-dependent expression (citrate, fructose, fucose, galactose, glucose, maltose, and xylose), while others increased gall expression levels (arabinose, glycerol, isocitrate, mannitol, mannose, pyruvate, succinate, and sucrose) (Table 3).

As a third parameter, the effect of the nitrogen source was tested. For the starting medium, the optimal conditions so far determined were used (MinA medium, pH 5.0, with 20 mM succinate). Whereas none of the tested amino acids alone could induce ORF1 expression (data not shown), all of them had a negative effect on the gall-dependent induction levels (Table 4). Interestingly, the combination of succinate and histidine gave rise to very high GUS activity.

The addition of plant or gall extracts to histidine and succinate resulted in an important decrease in the induction levels (Table 5). This observation prompted us to test whether these extracts contained compounds that repressed *fas* induction. To remove common plant metabolites, leafy gall extracts were used as a nutritional source for *E. coli*. After overnight growth, the *E. coli* cells were removed by centrifugation, and the extracts were filter sterilized and subsequently used in combination with histidine and succinate. Measurement of *fas* gene expression under these conditions showed that there was indeed a partial relief of the repression of the histidine and succinate induction levels observed with complete-plant and leafy gall extracts (Table 5).

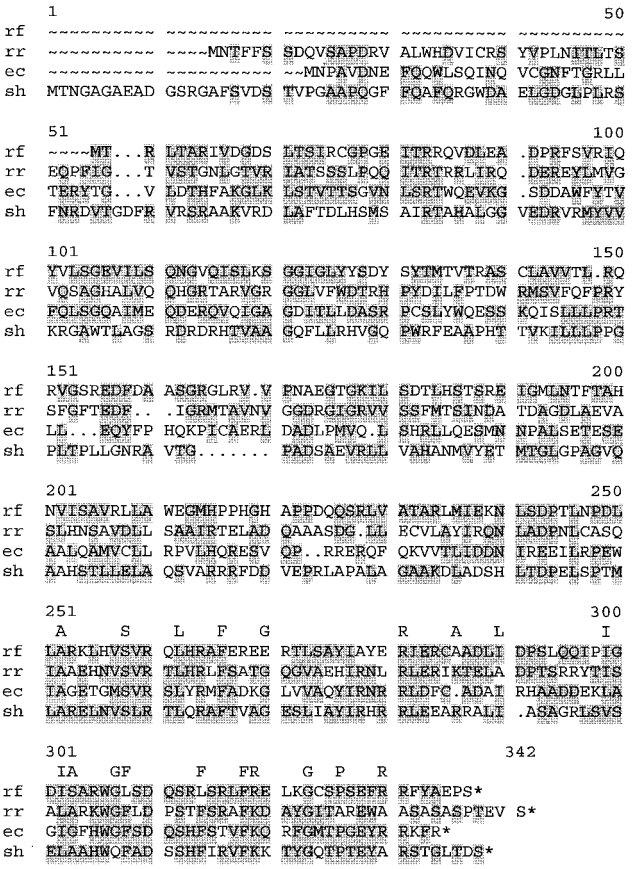

FIG. 2. Alignment of AraC-type transcriptional regulators of *R. fascians* (rf), *R. rhodochrous* (rr), *E. coli* (ec), and *S. hygroscopicus* (sh). Identical and/or similar amino acids are shaded. The AraC family characteristic motif is indicated above the alignment (21); with n representing any amino acid, it is as follows: $An_5Sn_3Ln_3Fn_4Gn_{10}Rn_3An_3Ln_8$ (I/V)n_2 (I/V)n_4 G(F/Y)n_5Fn_3F(R/K)n_3Gn_2P. Dots were inserted for optimal alignment, and the asterisks indicate stop codons.

Then, the influence of cell density on *fas* expression was investigated. Cultures were used with different optical densities at 600 nm (OD_{600}) at the start of the induction with histidine and succinate in MinA medium at pH 5.0. The results presented in Fig. 4C show a direct correlation between cell density and expression level. A similar result was obtained when leafy gall extracts were used in MinA medium at pH 5.0 (data not shown). Thus, the optimized conditions for *fas* gene expression are MinA medium at pH 5.0 supplemented with 20 mM succinate and 5 mM histidine and at a starting OD_{600} of 2.0.

Finally, *fas* expression was monitored under anaerobic and semianaerobic conditions. Optimized cultures (Fig. 4D) and cultures induced with leafy gall extracts (data not shown) were incubated under different oxygen concentrations. The experiment showed that low oxygen concentrations had a negative effect on *fas* expression.

The expression of *fasR* is constitutive. Because AraC-type transcriptional regulators are often autoregulatory (8, 25), a transcriptional fusion of the upstream region of *fasR* to *uidA* was constructed (pRFDV6) (Fig. 1D). Introduction of the replicating plasmid pRFDV6 into strain D188 and D188Δ*fasR* and incubation under the different conditions altering *fas* gene expression showed that the overall expression of *fasR* was con-

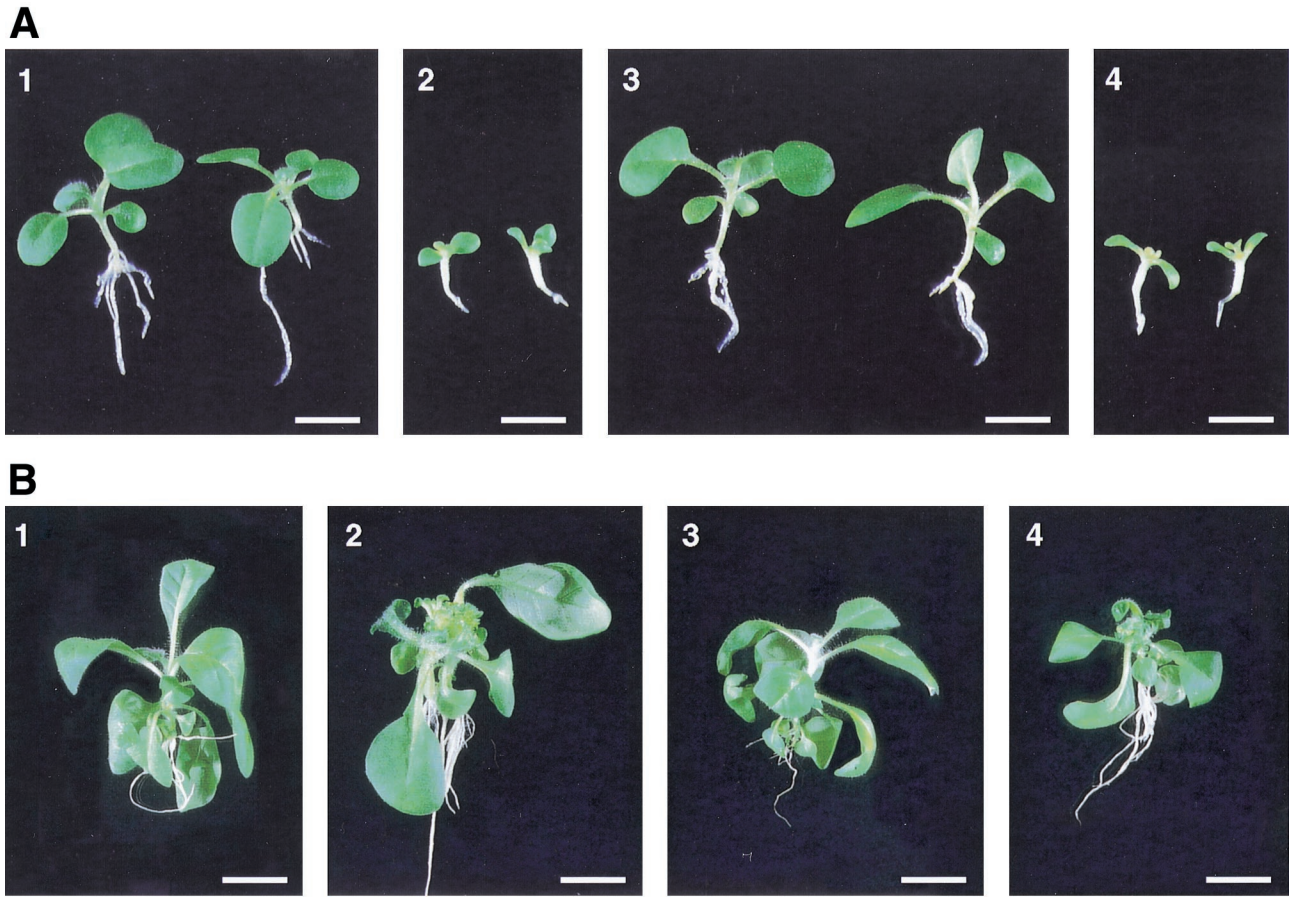

FIG. 3. Phenotypes of tobacco inoculated with different *R. fascians* strains. (A) Seedlings infected with D188-5 (1), D188 (2), D188Δ*fasR* (3), and D188Δ*fasR*(pRFDV2) (4). (B) Inoculation after decapitation of the apical meristem without bacteria (1), with strain D188 (a leafy gall forms at the cutting site and no axillary shoot meristem can grow out (2), with strain D188Δ*fasR* phenotype as in panel 1 (3), and with strain D188Δ*fasR*(pRFDV2) phenotype as in panel 2 (4). Bars = 0.5 cm (A) and 2 cm (B).

stitutive and comparable in the two strains (in strains D188 and D188Δ*fasR*, not induced [178.8 ± 21.1 and 144.8 ± 18.0, respectively] and induced with succinate and histidine [140.8 ± 18.0 and 116.3 ± 8.2, respectively]).

Transcription of the *fas* genes is affected by *fasR* and another pFiD188-encoded regulator. With the optimized induction conditions for *fas* gene expression set (see above), the possible regulatory role of *fasR* could be assessed. Because GUS activity from pJDGV5 is the result of the combined action of transcriptional and translational signals, transcriptional GUS fusions were constructed. The same upstream region as in pJDGV5 was fused to a *gus* gene carrying its own translational signals, resulting in plasmid pRFWT11 (Fig. 1C). The plasmid was introduced into strains D188, D188Δ*fasR*, and D188-5, which is a linear plasmid-free strain, and *fas* expression was determined. Under noninduced conditions, D188(pRFWT11) showed a GUS activity level comparable to that of the translational fusion under induced conditions (Table 6). Moreover, the transcriptional activity was not affected by the addition of gall extract, by histidine combined with succinate, by any of the tested carbon and nitrogen sources, or by the pH (data not shown). However, the level of transcription did increase with the cell density (data not shown).

In strain D188-5(pRFWT11), a significant decrease in transcriptional GUS activity was observed compared to the levels measured in D188. However, the transcriptional GUS activity seemed not to be dependent on *fasR*, as shown by the constitutively high *gus* expression level in strain D188Δ*fasR*(pRFWT11) (Table 6). These results indicate that other regulators involved in *fas* gene expression must be located on the linear plasmid pFiD188.

To evaluate the possible importance of the promoter copy number in regulation, the transcriptional *gus* expression was determined upon integration into the genome. For this purpose, a nonreplicating plasmid was constructed carrying the same GUS fusion as in pRFWT11. This plasmid, pJBWT2 (Fig. 1C), was introduced into D188 and D188Δ*fasR* via electroporation (12). By Southern hybridization analysis, the plasmid was found integrated into the genome of both strains via illegitimate integration (data not shown). In strain D188::pJBWT2, the measured transcription of the *fas* genes was again constitutive, although the absolute expression level was fivefold lower than that of the replicating transcriptional GUS fusion (Table 6). Furthermore, GUS activity in D188Δ*fasR*::pJBWT2 was another twofold lower (Table 6), indicating that FasR does affect *fas* gene transcription.

The environmental modulation of *fas* gene expression is translationally controlled and requires *fasR*. Considering the nonpathogenic phenotype of D188Δ*fasR* and the data described above, we hypothesized that *fasR* would be involved in the translational control of *fas* gene induction. Therefore, plasmid pJDGV5 carrying a translational ORF1-GUS fusion was

TABLE 3. Effects of carbon sources on ORF1 expression[a]

Carbon source (20 mM)	Growth	GUS activity	
		+ Plant extract (40 μl)	+ Leafy gall extract (20 μl)
YEB	+++	3.6 ± 0.3	3.0 ± 0.2
None	0	3.8 ± 0.5	29.6 ± 7.9
Glucose	+++	2.2 ± 0.4	36.7 ± 2.5
Fructose	+++	4.2 ± 3.9	51.8 ± 16.5
Sucrose	+++	5.4 ± 2.1	97.5 ± 28.7
Maltose	0	3.2 ± 0.9	38.1 ± 4.6
Mannitol	+++	5.9 ± 3.0	86.6 ± 5.8
Glycerol	++	2.6 ± 0.6	75.1 ± 3.7
Galactose	0	3.4 ± 1.0	38.2 ± 18.1
L-Arabinose	+++	2.6 ± 0.2	79.1 ± 12.1
D-Arabinose	0	2.3 ± 0.3	35.3 ± 12.5
Fucose	0	3.1 ± 0.5	36.5 ± 13.1
Mannose	+++	3.2 ± 0.6	96.0 ± 31.2
Xylose	0	2.4 ± 0.3	59.3 ± 24.4
Succinate	0	2.5 ± 0.2	89.6 ± 6.1
Citrate	0	4.1 ± 3.5	43.5 ± 3.1
Isocitrate	0	3.1 ± 0.1	85.4 ± 25.1
Malate	−	2.1 ± 0.1	5.3 ± 0.7
Pyruvate	0	2.5 ± 0.2	53.2 ± 7.6
α-Ketoglutarate	−	4.2 ± 0.9	4.8 ± 0.3
Glucolate	−	4.8 ± 1.0	4.6 ± 0.4
Glyoxylate	−	1.8 ± 0.2	4.3 ± 0.5
Fumarate	−	3.8 ± 0.5	5.6 ± 0.7

[a] The data are averages of three independent experiments and were measured with test strain D188(pJDGV5) in MinA medium at pH 5.0. For details, see Materials and Methods. +, ++, +++, growth relative to the control condition without addition of any carbon source (0); −, negative effect of the added carbon source on bacterial growth. For details, see Materials and Methods.

introduced into strains D188Δ*fasR* and D188-5. Measurement of the GUS activity showed that the *fas* genes could not be induced in either of the two strains (Table 6). This observation indicates that FasR is essential for regulated *fas* gene expression and that the environmental regulation must be exerted by a translational regulator that is under the control of *fasR*. The possible role of the promoter copy number was assessed with the integrating plasmid pUCWT1 (Fig. 1C) carrying the same GUS fusion as in pJDGV5. The data in Table 6 show that upon integration of the GUS fusion, the translational expression pattern was retained: in strain D188::pUCWT1, succinate combined with histidine led to the induction of the *fas* genes, and no induction could be obtained in strain D188Δ*fasR*::pUCWT1.

DISCUSSION

We have characterized a regulatory gene in *R. fascians*, *fasR*, that belongs to the AraC family of transcription regulators (Fig. 2) (22) and proves to be essential for leafy gall formation (Fig. 3). AraC-type regulators have been shown to regulate virulence genes in the gram-negative phytopathogens *Ralstonia solanacearum* (23), *Pseudomonas syringae* pv. phaseolicola (69), and *Xanthomonas campestris* (63), as well as in several animal pathogens (16, 30, 47, 54, 62). Typically, the regulatory characteristics exerted by this class of proteins are very complex, with the regulators acting as transcriptional activators or repressors, depending on the growth conditions, their cellular concentrations, the relative positions of their binding sites in the promoters they regulate, and the presence of particular signals. Because a *fasR* deletion mutant, D188Δ*fasR*, is nonpathogenic and exhibits the same phenotype on plants as a *fas* mutant, it was hypothesized that FasR would regulate *fas* gene expression.

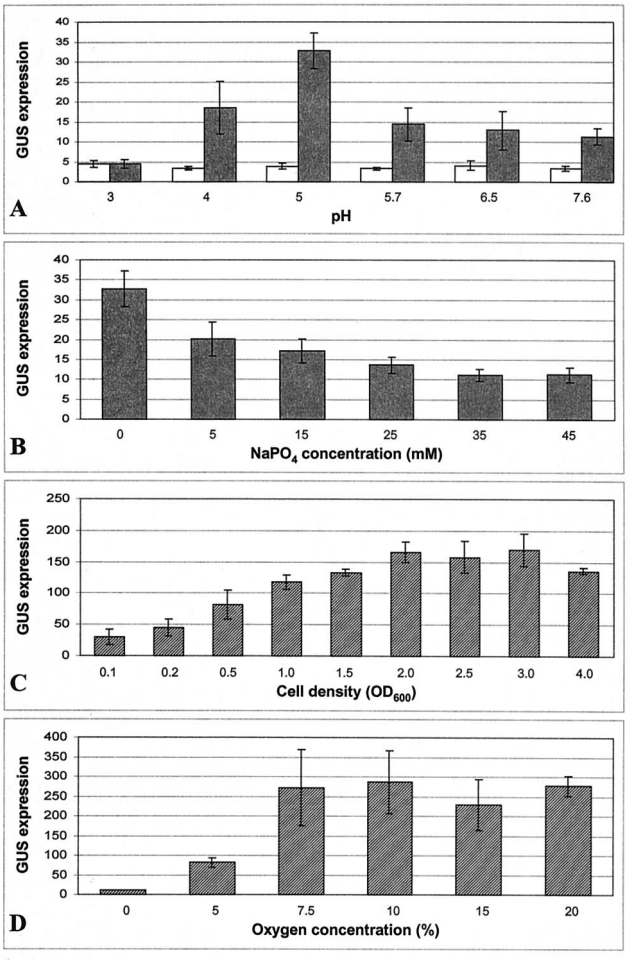

FIG. 4. Effects of different conditions on ORF1 expression as measured with test strain D188(pJDGV5). The effects of pH (A), phosphate (B), cell density (C), and O_2 concentration (D) in MinA medium with glucose (A and B) and with 5 mM histidine and 20 mM succinate (C and D) at pH 5.0 (B, C, and D) and at OD_{600} (D) with plant extract (open bars), with leafy gall extract (shaded bars), and without extract (hatched bars) are shown. The error bars indicate standard deviations.

To obtain higher expression levels in batch culture, several parameters had to be adjusted. As a result, *fas* gene expression could be induced upon addition of leafy gall extract, but the highest expression level was obtained in MinA medium at pH 5.0 (Fig. 4A) to which a combination of succinate and histidine was added (Table 5) and with an initial starting OD_{600} of 2.0 (Fig. 4C). The observed pH optimum is not surprising, because plant fluids are slightly acidic and high *fas* gene expression under these conditions would enable interference with the development of the plant. In other pathogens, virulence gene expression is often correlated with the pH conditions met in the host (36, 48, 53).

Several carbon sources had a positive effect on the gall-dependent induction levels. Some of these carbon sources (arabinose, fructose, glucose, glycerol, mannitol, mannose, and sucrose) also had a promoting effect on bacterial growth (Table 3). Because there is a positive correlation between cell density and induction level (Fig. 4C), the observed effect of these compounds might be mediated via cell growth. Nevertheless, other carbon sources (isocitrate, pyruvate, and succinate) had no promoting effect on bacterial growth but still

TABLE 4. Effects of amino acids on gall-induced ORF1 expression[a]

Amino acid(s) (5 mM)	GUS activity	
	+ Plant extract (40 μl)	+ Leafy gall extract (20 μl)
None	2.5 ± 0.7	37.4 ± 5.4
Glutamic acid	3.0 ± 1.8	25.0 ± 2.6
Leucine	2.7 ± 1.0	4.4 ± 0.9
Threonine	2.4 ± 0.6	9.6 ± 2.6
Asparagine	2.5 ± 0.6	3.2 ± 1.5
Casamino Acids	1.6 ± 0.4	1.3 ± 0.4
Arginine	2.7 ± 1.0	2.3 ± 1.4
Lysine	2.7 ± 0.7	1.9 ± 0.2
Tyrosine	3.0 ± 1.2	2.0 ± 0.9
Histidine	35.2 ± 16.5	17.9 ± 5.1

[a] The data are averages of three independent experiments and were measured with test strain D188(pJDGV5) in MinA medium at pH 5.0 supplemented with 20 mM succinate. For details, see Materials and Methods.

augmented gall-dependent induction levels (Table 3). These carbon sources are Krebs cycle intermediates, which might be related to the function of the *fas*-encoded proteins. In this respect, our working model states that part of the *fas* operon constitutes an electron transport chain that delivers high-energy electrons for the cytochrome P450 reaction (24). The presence of the Krebs cycle intermediates might signal that the substrates for P450 activity are available and, in a dual function, lead to the stronger induction of the *fas* operon. Alternatively, in the acetosyringone-mediated induction of the *vir* genes of *Agrobacterium tumefaciens*, several monosaccharides exhibit a synergistic effect (6, 52). A similar observation has been made for the phenolic-induced expression of the *syrB* gene of *P. syringae* pv. syringae (40). In the case of the leafy gall extract-mediated *fas* gene expression in *R. fascians*, the carbon sources could have an analogous function. The observation that a combination of histidine and succinate also strongly induces *fas* gene expression is puzzling. Possibly, both leafy gall extracts and histidine-succinate provoke a specific metabolic state of the bacteria in which *fas* gene expression is high. In this hypothesis, such conditions would not prevail in plant extracts.

Interestingly, histidine also induces *fas* gene expression in combination with the carbon sources that do not promote bacterial growth but that are synergistic on the leafy gall-dependent induction levels (Table 3). As a corollary, histidine could be hypothesized to be an actual inducing factor present in leafy gall extracts. Preliminary amino acid analysis of uninfected and infected plant tissues did not reveal an apparent increase in histidine levels upon infection with *R. fascians* (data not shown); nevertheless, histidine might be a functional analogue of a putative inducing factor. To date, no further data are available to favor any of these hypotheses.

TABLE 5. Presence of repressing compounds in extracts affecting ORF1 expression induced by succinate (20 mM) and histidine (5 mM)[a]

Compound	GUS activity
Succinate	1.8 ± 0.5
Succinate + histidine	149.3 ± 34.1
Succinate + histidine + plant extract	35.2 ± 6.5
Succinate + histidine + gall extract	17.9 ± 5.1
Succinate + histidine + depleted gall extract	52.8 ± 8.1

[a] The data are averages of three independent experiments and were measured with test strain D188(pJDGV5) in MinA medium at pH 5.0. For details, see Materials and Methods.

The higher expression levels obtained at higher cell densities (Fig. 4C) might at first sight resemble quorum sensing. However, *fas* gene expression can also be induced at low cell densities, and the expression levels gradually increase with cell density. These data indicate that the increased expression of the *fas* genes functions via a mechanism that differs from the cell density-dependent expression of LuxR-LuxI-homologous systems, in which a critical cell density is required (20, 45). A possible explanation for our results is that a higher cell density or the presence of some carbon sources alters the metabolism or the physiological state of the bacteria, rendering them more prone to express the *fas* genes.

Besides the positive effects of carbon sources and cell density, phosphate and amino acids had a negative influence on gall-dependent *fas* gene expression (Fig. 4B and Table 4). In *A. tumefaciens*, a similar, albeit more drastic, effect of phosphate was observed for *virG* expression (64). Because phosphate is often very scarce in nature, its limitation could be a signal for the bacterium to interact with the plant to produce galls that may serve as phosphate sources. Crude plant and leafy gall extracts proved to repress the high induction levels obtained with histidine and succinate. Removal of general metabolites from leafy gall extracts by depletion with *E. coli* partially relieved this inhibitory effect (Table 5). The inhibitory activity of gall extracts on histidine and succinate induction could be interpreted as a result of catabolite repression. Inhibition of gene expression by nitrogen sources has been reported in *Bacillus subtilis* (1, 19); in these cases, the mechanism involves regulation of transcription initiation (67). We have shown that several general amino acids inhibit *fas* gene induction by gall extracts (Tables 4 and 5) or by histidine and succinate (data not shown). Because gall and plant extracts represent a rich mixture of several general metabolites, such catabolite repression could account for the lower expression levels obtained by combining histidine and succinate with these extracts. Following overnight growth of *E. coli* on such extracts, the resulting depletion of metabolites can be assumed to relieve catabolite repression, which could explain the higher *fas* gene expression levels obtained by combining histidine and succinate with such depleted extracts.

To unravel the regulatory circuits controlling the induction of the *fas* genes, translational and transcriptional GUS fusions to ORF1 were constructed, on both replicating and integrating plasmids (Fig. 1C and Table 2), and the expression patterns were determined in strain D188, the plasmid-free strain D188-5, and strain D188Δ*fasR*. With the replicating transcriptional fusion in strain D188, *fas* expression was constitutive independently of the pH and carbon or nitrogen sources and 30-fold higher than that measured with the translational fusion under noninducing conditions (Table 6). This result shows that under noninducing conditions translation is repressed and that *fas* gene expression is controlled at the translational level. Comparison of the transcription levels in strains D188 and D188-5 further suggested that a second transcriptional regulator besides FasR is located on pFiD188. Integration of the transcriptional fusion into the genome of strains D188 and D188Δ*fasR* resulted in lower expression levels and showed that FasR also had a positive effect on *fas* gene transcription. The fact that this result was not observed when the replicating plasmid was used suggests that the effect of the regulatory protein is titrated out because of multiple copies of the *fas* promoter. For the translational fusions, similar results were obtained with the replicating and integrated constructs. This observation could be explained by assuming that one or more *trans*-acting factors that are involved in translational regulation are present in limiting amounts only. In strain D188, *fas* gene

TABLE 6. Expression of ORF1 as measured with different test strains[a]

Strain and condition	pRFWT11 (replicating transcriptional)	pJBWT2 (integrated transcriptional)	pJDGV5 (replicating translational)	pUCWT1 (integrated translational)
D188/S	155.3 ± 15.6	31.5 ± 4.0	4.6 ± 0.2	6.0 ± 0.2
D188/SH	142.2 ± 25.8	30.8 ± 0.6	158.4 ± 8.0	158.4 ± 8.3
D188ΔfasR/S	162.3 ± 29.7	17.3 ± 1.6	4.1 ± 0.2	7.2 ± 0.3
D188ΔfasR/SH	131.0 ± 28.3	16.7 ± 2.6	3.9 ± 0.5	7.3 ± 0.4
D188-5/S	60.9 ± 30.4	ND	6.5 ± 1.5	ND
D188-5/SH	61.1 ± 33.9	ND	6.2 ± 1.0	ND

[a] In MinA medium at pH 5.0 and a starting OD_{600} of 2.0. /S, not-induced condition with the addition of 20 mM succinate; /SH, induced condition with the addition of 20 mM succinate and 5 mM histidine; ND, not determined. For details, see Materials and Methods.

expression was induced and modulated by environmental factors. However, in strain D188-5 and D188Δ*fasR*, no induction could be obtained (Table 6). Together, these results indicate that *fas* gene expression is subject to a complex regulatory network incorporating different regulatory loci acting at the transcriptional and translational levels. Thus, the phytopathogen can cope with the variable conditions that it encounters during interaction with its host plant. In this regulatory network, *fasR*, which encodes a transcriptional regulator, plays a crucial role in the induction of *fas* gene expression, which is modulated at the posttranscriptional level. The mechanism of this regulation is currently unknown, but it could be the result of a modulation of RNA or protein stability or of translation initiation. Whatever the mechanism, the factors that control it have to be themselves under control of the *fasR* gene, either directly or indirectly.

Based on the data obtained we can propose a working model for the regulation of *fas* gene expression. In this model, the induction of gene expression is controlled at the translational level and requires FasR. The translational regulator is encoded by the linear plasmid, and its transcription is regulated by FasR. The induction of the *fas* genes is probably mediated by the interaction of one or more inducing compounds present in infected plant tissue with the translational regulatory protein or with FasR. Furthermore, FasR activates *fas* gene transcription. Finally, a second transcriptional activator of the *fas* genes is present on the linear plasmid. Although the majority of regulatory networks, which often control very complex processes in bacteria, consist of only transcriptional regulators (3, 46), the interplay of transcriptional and translational regulators that direct the expression of specific pathways has been reported (32). The regulation of *fas* gene expression is another example of the latter. Based on the low G+C content of *fasR* and on the apparently superimposed function of FasR on other regulatory pathways, we speculate that *fasR* might have been acquired relatively late during the evolution of *fas* gene regulation in *R. fascians*.

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